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# THE ROLE OF SOLVENT IN THE STABILIZATION OF HELICAL STRUCTURE: THE LOW pH RIBO $A_8$ AND $A_{10}$ DOUBLE HELICES IN MIXED SOLVENTS *

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The order-disorder transitions of the double helices formed by the ribo-oligoadenylic acids $rA_8$ and $rA_{10}$ at pH 4.2 have been investigated in a series of organic/aqueous mixed solvents. Melting temperature data, $T_m$, derived from the uv melting curves were used to define the stability of the double helices in the different mixed solvent systems. It was found that the extent of helix destabilization depended in a predictable fashion on both the quantity and the nature of the added organic solvents. For the $C_1$ through $C_4$ aliphatic alcohols, the longer, less branched alcohols proved to be more effective destabilizers of the helical structure. Significantly, the amides proved to be more powerful destabilizers than the alcohols. Analysis of the melting curves provided the Van't Hoff enthalpy change for each transition. The data are interpreted in terms of the role of solvent in the stabilization of ribonucleic acid structure.

## 1. Introduction

Experimental investigations of the helix to coil transitions of DNA's in mixed solvent systems has provided much useful information concerning the forces that stabilize DNA structure [1–5]. In contrast, practically all physical studies on RNA molecules have been carried out in neutral, aqueous buffer solutions. This is unfortunate since a great deal of RNA biochemistry occurs in environments that differ greatly from neutral, aqueous buffer conditions. For example, the large number of chemical events that take place at the surface of or inside of cell membranes cannot be viewed as occurring in aqueous environments. On the contrary, these processes occur in highly nonpolar surroundings. Insofar as pH is concerned, recent studies reveal that cells can contain vesicles which possess pH environments as low as 5. In this connection, Bina-Stein and Crothers [6] have shown that t-RNA molecules can assume a variety of different structures at pH values between 4.5 and 7.

Observations such as these clearly demonstrate that restriction of our attention to neutral, aqueous buffer solutions may well cause us to overlook a host of biologically significant RNA structures and their associated biochemical functions. In addition, confining our attention to such narrow solvent conditions prevents us from obtaining useful new information concerning the role of solvent in the stabilization of RNA structure.

In order to alleviate this situation, we have initiated a program in which different ribonucleic acids are studied under a variety of less traditional solution conditions. In this paper we report the results obtained from an investigation of the effect of added organic solvents on the stability of the double helices formed by $rA_8$ and $rA_{10}$ at pH 4.2.

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## 2. Experimental section

### 2.1. Materials and methods

The $(Ap)_7A$ and $(Ap)_9A$ used in these studies were obtained from Boehringer Mannheim. The purity of each sample was confirmed by chromatography. All the organic solvents used to make up the mixed solvent systems were spectro grade and obtained through Fischer Scientific. The aqueous buffer consisted of 0.1 M NaCl and 0.01 M sodium acetate adjusted to pH 4.2.

The concentrations of the oligomers were spectrophotometrically determined using the extinction coefficients reported by Eigen and Pörschke [7]. These values were confirmed by alkaline hydrolysis of the oligomers and use of the known monomer extinction coefficients to recalculate the concentrations.

The uv melting curves reported here were measured at 259 nm using a temperature programmable, thermoelectrically controlled, automatic recording spectrophotometer (Perkin-Elmer Corporation). The temperature was increased continuously at a rate of 0.5 deg C/min. Prior to each melting experiment wavelength scans were carried out at 5, 25 and 65°C in order to characterize the samples and to disrupt any aggregates. The expected hyperchromicity was observed with a shift in the extinction maximum from 252 to 257 nm [7]. Repetitive runs revealed the melting temperature data derived from the uv melting curves to be reproducible to ± 0.5°C.

The formamide and DMF mixed solvent "buffers" exhibited a considerable temperature-dependent background at 259 nm. Thus, melting curves for these solvent systems were carried out at 270 nm. To justify comparison of these data with those obtained at 259 nm, it was shown that identical melting temperatures and Van 't Hoff enthalpies could be obtained by monitoring the other transitions at either 259 or 270 nm.

### 2.2. Treatment of the data

In order to derive Van 't Hoff enthalpy values from the optical data, the experimental absorbance versus temperature curves were converted into $\alpha$ versus $T$ melting curves, where $\alpha$ represents the fraction in single strand. This conversion was accomplished by assuming that the fractional change in absorbance at any temperature is proportional to the extent of reaction at that temperature. Thus a value of $\alpha$ can be obtained, at any temperature by simply taking the ratio of the distance between the two baselines and the distance between the lower baseline and the experimental curve. In such a treatment, the melting temperature ($T_m$) is defined as the temperature at which $\alpha$ equals 0.5. Evaluation of the resulting $\alpha$ versus $T$ curve allows one to calculate the Van 't Hoff enthalpy change for the transition. The details are described below.

When two strands of a self-complementary molecule (i.e. $A_8^+$) combine in an all-or-none fashion to form a fully bonded helix, the equilibrium constant may be written as

$$K = \frac{\alpha/2}{(1-\alpha)^2 C_T}, \tag{1}$$

where $C_T$ is the total strand concentration and $\alpha$ represents the degree of conversion of the initial into the final state.

The variation of this equilibrium constant with temperature provides a means for determining the Van 't Hoff enthalpy ($\Delta H_{v.H.}$)

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H_{v.H.}}{RT^2}. \tag{2}$$

Eqs. (1) and (2) can be combined to yield the following expression for the Van 't Hoff enthalpy change accompanying a helix-coil transition for self-complementary strands.

$$\Delta H_{v.H.} = 6RT_m^2 (\partial\alpha/\partial T)_{T_m}. \tag{3}$$

Thus, if the slope of an $\alpha$ versus $T$ plot is evaluated at the melting temperature, the Van 't Hoff enthalpy change accompanying the transition can be calculated from eq. (3).

## 3. Results

### 3.1. Solvent-induced destabilization

#### 3.1.1. Double stranded base stacking

Figs. 1a and 1b show families of melting curves for the thermally induced order-disorder transition of $rA_{10}$ at pH 4.2 in a series of 5 and 10% mixed solvent systems. Table 1 summarizes the melting temperature, $T_m$, data obtained by analysis of these and other curves

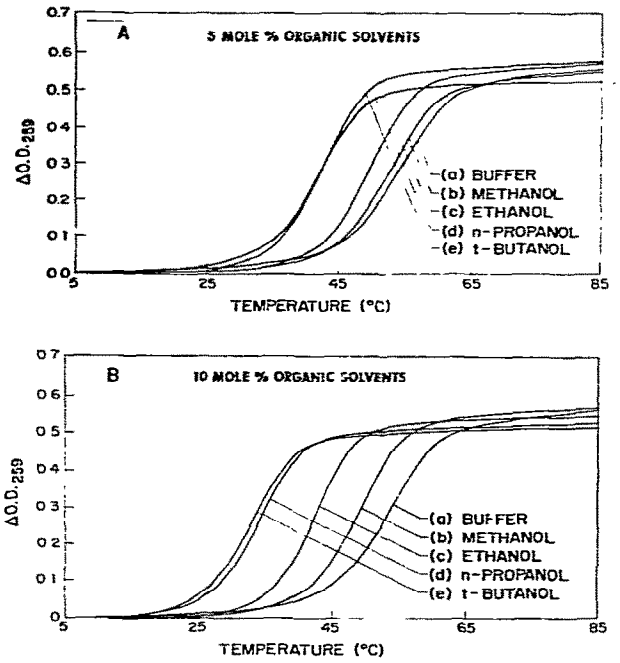


Fig. 1. Absorbance change versus temperature for $rA_{10}$ at pH 4.2 in the 5 mole percent (A) and the 10 mole percent (B) mixed solvent systems.

for both $rA_8$ (at one concentration) and $rA_{10}$ (at two concentrations).

Several significant trends should be noted.

a) At comparable mole fractions, the longer straight-chain alcohols, when compared with their shorter homologs, prove to be more effective destabilizers of the double helical structure. Thus, the $T_m$'s decrease as one reads down a column in table 1.

b) For a given organic solvent, the ability to destabilize the helix increases as the mole fraction increases even up to 15 mole percent. That is to say, no saturation or leveling off was observed for the solvent-induced destabilization. Thus, for a given helix, the $T_m$'s decrease as one reads across a row in table 1.

c) Branching of the chain appears to reduce the destabilizing effect of the added organic solvent relative to its straight-chain isomer. Thus, isopropanol is slightly less destabilizing than n-propanol.

d) The amides are more powerful destabilizers of the helical structure than the alcohols.

Table 1
Melting temperature, $T_m$, for $rA_8$ and $rA_{10}$ in mixed solvent systems at pH 4.2

| Solv Solvent system | $rA_8$ (7.8 μm) a) $T_m$(°C) 5% | 10% | $rA_{10}$ (5.5 μm) a) $T_m$(°C) 5% | 10% | 15% | $rA_{10}$ (6.5 μm) a) $T_m$(°C) 5% | 10% |
|---|---|---|---|---|---|---|---|
| Buffer | 35.0 | 35.0 | 45.8 | 45.8 | 45.8 | 53.3 | 53.3 |
| MeOH | 34.0 | 32.0 | 43.4 | 41.0 | 39.5 | 51.2 | 49.2 |
| EtOH | 32.0 | 27.5 | 41.9 | 34.4 | 28.0 | 49.2 | 44.4 |
| i-PrOH | 28.0 | 19.0 | 35.8 | 25.0 | – | – | – |
| n-PrOH | 25.0 | 17.0 | 34.5 | 23.9 | 17.3 | 42.2 | 34.4 |
| t-BuOH | 25.0 | 14.5 | 34.5 | 21.7 | 19.5 | 41.4 | 33.0 |
| Formamide | 24.0 | 15.0 | – | – | – | | |
| DMF | 21.0 | 7.0 | 30.5 | 17.2 | – | | |

a) Concentrations given in moles of double strand.

The trends described above are shown graphically in figs. 2a and 2b, and tabulated in table 2. Several quantitative features of these data deserve further comment. An examination of the $\Delta T_m$ columns in table 2 (or inspection of fig. 1) reveals that a 5% increase in the mole fraction of a given organic solvent exerts a destabilizing effect that is approximately equivalent to the increment observed for the addition of one more non-branched methylene group to the straight-chain alcohol. Thus, the destabilizing influence ($\Delta T_m$ in table 2) of 10% methanol (relative to buffer) is essentially equivalent to that of 5% ethanol.

Inspection of the last column in table 2 reveals that the ratio $\Delta T_m(10\%)/\Delta T_m(5\%)$ is approximately constant and equal to $2.0 \pm 0.3$. This indicates that the destabilization effect of any given organic solvent is simply a linear additive function of its mole fraction. In this connection it should be mentioned that the destabilization caused by a 10% organic solvent solution (composed of 5% methanol and 5% ethanol) is approximately the average of the destabilization caused by two 10% solutions of the corresponding pure organic solvents. This further indicates that the destabilization effects are simply additive.

The third column in table 2 lists the slopes of the lines shown in figs. 2a and 2b. The magnitude of these slopes reflect the destabilizing ability of each organic solvent. Thus, as one proceeds down the list of solvents in table 2 (from methanol to DMF) one finds a steady increase in $\partial T_m/\partial M$ from approximately −0.4 to −2.8.

Table 2
Solvent induced changes in the melting temperature of $rA_8$ and $rA_{10}$ at pH 4.2

| Solvent system | $rA_8$ (7.7 μm) | | | | $rA_{10}$ (5.5 μm) | | | | $rA_{10}$ (6.5 μm) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\Delta T_m$ [a] | | $\left(\frac{\partial T_m}{\partial M}\right)$ [b] | $\frac{\Delta T_m(10\%)}{\Delta T_m(5\%)}$ | $\Delta T_m$ [a] | | $\left(\frac{\partial T_m}{\partial M}\right)$ [b] | $\frac{\Delta T_m(10\%)}{\Delta T_m(5\%)}$ | $\Delta T_m$ [a] | | $\left(\frac{\partial T_m}{\partial M}\right)$ [b] | $\frac{\Delta T_m(10\%)}{\Delta T_m(5\%)}$ |
| | 5% | 10% | | | 5% | 10% | | | 5% | 10% | | |
| MeOH | -1.0 | -3.0 | -0.3 | 3.0 | -2.4 | -4.8 | -0.5 | 2.0 | -2.1 | -4.1 | -0.4 | 2.0 |
| EtOH | -3.0 | -7.5 | -0.8 | 2.5 | -3.9 | -11.4 | -1.2 | 2.9 | -4.1 | -8.9 | -0.9 | 2.2 |
| i-PrOH | -7.0 | -16.0 | -1.6 | 2.3 | -10.1 | -20.8 | -1.8 | 2.1 | – | – | – | – |
| n-PrOH | -10.0 | -18.0 | -1.9 | 1.8 | -11.3 | -21.9 | -1.9 | 1.9 | -11.1 | -18.9 | -1.9 | 1.7 |
| t-BuOH | -10.0 | -20.5 | -2.1 | 2.0 | -11.3 | -24.1 | -2.4 | 2.1 | -11.9 | -20.3 | -2.1 | 1.7 |
| Formamide | -11.0 | -20.0 | -2.1 | 1.8 | – | – | – | – | | | | |
| DMF | -14.0 | -28.0 | -2.8 | 2.0 | -15.3 | -28.6 | -2.9 | 1.9 | | | | |

a) $\Delta T_m = T_m(\text{org}) - T_m(\text{buffer})$.
b) $\partial T_m/\partial M$ = change in melting temperature with change in mole percent of the indicated organic solvent.

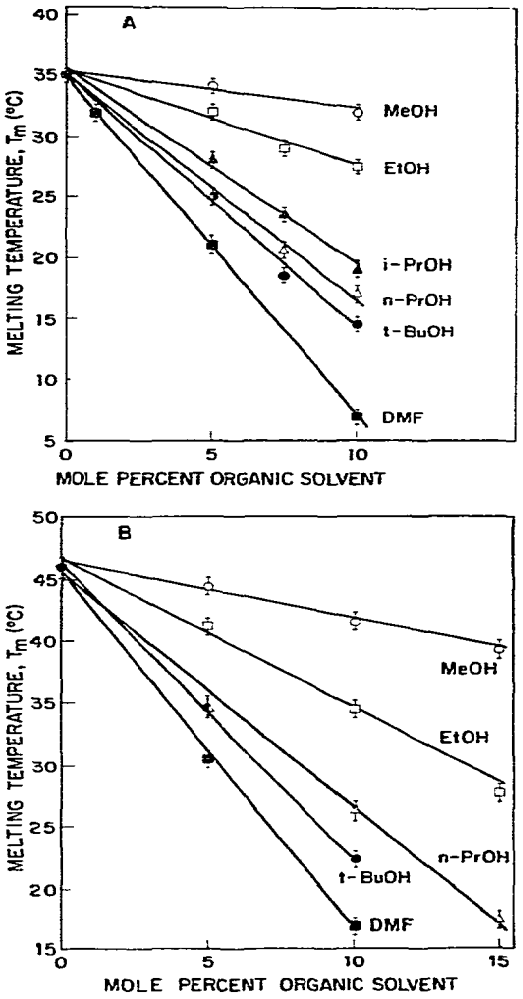


Fig. 2. The melting temperatures, $T_m$, versus the mole percent of the indicated organic solvents for $rA_8$ (A) and $rA_{10}$ (B).

The reasonably good agreement between the $\partial T_m/\partial M$ values obtained for both the $rA_8$ and the $rA_{10}$ helices would appear to indicate that the solvent induced destabilization exhibits no significant dependence on the chainlength of the oligomer.

These trends are all consistent with an interpretation in which the destabilization of the double helix is caused by a non-specific solvent effect resulting from

an increase in the nonpolar nature of the environment surrounding the helix. This will be discussed more completely in a later section.

It should be noted that in interpreting our $T_m$ data we are not unwittingly comparing different double helices in the different solvent systems. This is confirmed by studies which reveal that changes in the solvent composition do not affect the shape of the titration curve [8]. Thus, our $T_m$ data do not refer to different initial states (double helices) in the different mixed solvents.

*3.1.2. Single-stranded base stacking*

It is of interest to note the different final slopes in the O.D. versus $T$ curves shown in figs. 1a and 1b. These final slopes are generally associated with residual base stacking in the separated single strands [9,10]. One usually finds that the higher the melting temperature for a cooperative transition, the flatter the upper baseline due to thermal disruption of the single-stranded base stacking at the elevated final temperature [10]. However, inspection of the melting curves shown here reveals that the upper baselines become progressively flatter as the melting temperature decreases. This trend can be interpreted in terms of the effect of the added organic solvents on the base stacking in the separated single strands. The pure buffer exhibits the greatest final slope while increasing either the quantity of the added organic solvent or its hydrocarbon content serves to progressively flatten the final baseline. In other words, decreasing the "aqueous character" of the solvent serves to destabilize single-stranded base stacking. Significantly, the order of solvent denaturing effectiveness for the single-stranded base stacking parallels that observed for disruption of the double helical structure.

*3.2. Thermodynamic parameters*

*3.2. The enthalpy change*

Table 3 summarizes the enthalpy data obtained for the pH 4.2 order-disorder transitions of $rA_8$ and $rA_{10}$ in pure buffer and the mixed solvents. The method of analysis used to obtain these data has been described in the experimental section.

The data reveal no obvious correlation between the enthalpy change and the solvent-induced decrease in the melting temperature. In fact, the fluctuations in

Table 3
Enthalpy changes accompanying the helix-coil transitions of $rA_8$ and $rA_{10}$ at pH 4.2 in a series of mixed solvent systems

| | $rA_8$ (7.8 μm) | | $rA_{10}$ (5.5 μm) | | $rA_{10}$ (6.5 μm) | |
|---|---|---|---|---|---|---|
| Solvent system | $T_m$(°C) | $\Delta H_{v.H.}$ | $T_m$(°C) | $\Delta H_{v.H.}$ | $T_m$(°C) | $\Delta H_{v.H.}$ |
| Buffer | 35.0 | 63 | 45.8 | 81 | 53.3 | 70 |
| 5% MeOH | 34.5 | 56 | 43.4 | 76 | 51.2 | 84 |
| 5% EtOH | 32.0 | 59 | 41.9 | 91 | 49.2 | 68 |
| 5% i-PrOH | 28.0 | 58 | 53.8 | 87 | – | – |
| 5% n-PrOH | 25.0 | 74 | 34.5 | 83 | 42.2 | 74 |
| 5% t-BuOH | 25.0 | 60 | 34.5 | 89 | 41.4 | 80 |
| 5% Formamide | 24.0 | 54 | – | – | | |
| 5% DMF | 21.0 | 52 | 30.5 | 70 | | |
| 10% MeOH | 32.0 | 56 | 41.0 | 83 | 49.2 | 70 |
| 10% EtOH | 27.5 | 72 | 34.4 | 85 | 44.4 | 95 |
| 10% i-PrOH | 19.0 | 58 | 25.0 | 70 | – | – |
| 10% n-PrOH | 17.0 | 53 | 23.9 | 83 | 34.4 | 83 |
| 10% t-BuOH | 14.5 | 53 | 21.7 | 89 | 33.0 | 87 |
| 10% Formamide | 15.0 | 53 | – | – | | |
| 10% DMF | 7.0 | 53 | 17.2 | 83 | | |
| Average $\Delta H_{v.H.}$ (total) | | 58 ± 2 | | 82 ± 2 | | 79 ± 3 |
| $\Delta H_{v.H.}$ per stack | | 8 | | 9 | | 9 |

the enthalpy data may simply reflect the difficulties inherent in the Van 't Hoff analysis [13]. Nevertheless, it is of interest to note that the average enthalpy values of 8 kcal (mole of stack)$^{-1}$ for $rA_8$ and 9 kcal (mole of stack)$^{-1}$ for $rA_{10}$ are in excellent agreement with previously published values [7,9].

*3.2.2. The entropy change*

Calculation of the entropy change per mole of stacking interaction requires knowledge of the melting temperature of the corresponding polymer (pH 4.2 poly A) under comparable solvent conditions. Since the pH 4.2 poly A double helix has never been studied in organic/aqueous mixed solvents, our analysis is limited to the 100% aqueous buffer data reported here.

The general method uses oligomer and polymer melting temperature data and involves a simple Van 't Hoff treatment with a temperature-independent enthalpy change. The appropriate equation is

$$\log s_{T_m} = \frac{\Delta H}{2.3R}(1/T_m - 1/T_{m\infty}), \quad (4)$$

where $s$ represents the equilibrium constant for formation of a base-pair between two single strands in an already existing helix. For this calculation, the $T_m$ of the poly A double helix in pH 4.2 citrate buffer is taken to be $T_{m\infty} = 88.2°C$ [11,12] and the enthalpy changes are assigned the average buffer Van 't Hoff values reported here; that is, 9.0 kcal (mole of stack)$^{-1}$ for $rA_8$ and 8.4 kcal (mole of stack)$^{-1}$ for $rA_{10}$. The $s$ values obtained from this calculation are then used to calculate the free energy change accompanying the formation of a single base-pair in accordance with the equation

$$\Delta G^0 = -RT \ln s. \quad (5)$$

These $\Delta G^0$ values are then used in conjunction with the Van 't Hoff enthalpies reported earlier to calculate per base-pair entropy changes using the standard thermodynamic equation $\Delta G^0 = \Delta H^0 - T\Delta S^0$.

This treatment results in $\Delta S$ values per base pair disrupted of 23.2 and 24.6 cal deg$^{-1}$ mol$^{-1}$ for $rA_8$ and $rA_{10}$, respectively.

## 4. Discussion

Although it would be of interest to compare the results reported here with previously published data, none of the earlier studies on the low pH double helix of ribo-oligoadenylic acids have been carried out in mixed solvent systems. However, it is possible to compare the results obtained in this work in the 100% aqueous buffer system with previously published data.

### *4.1. Enthalpy change*

Eigen and Pörschke [7] used uv melting curves to investigate the pH 4.2 order-disorder transitions of $r(A)_N$ where $N$ varied from 6 to 10. They report a chainlength-dependent enthalpy of 8.6 kcal (mole of stack)$^{-1}$ for $rA_8$ and 9.9 kcal (mole of stack)$^{-1}$ for $rA_{10}$. Applequist and Damle [9] analyzed the hypochromicity data of Fresco, Blake and Doty on a series of ribo-oligoadenylic acids at pH 4.0 and report the enthalpy values per mole of stacking interaction to be 8.0 kcal and 9.8 kcal for $rA_8$ and $rA_{10}$, respectively. As inspection of table 3 reveals, our data are in reasonably good agreement with both of these previously reported studies.

### *4.2. Entropy*

The entropy change per mole of base-pair calculated by the thermodynamic treatment described above provides values of 23.2 cal deg$^{-1}$ mol$^{-1}$ for $rA_8$ and 24.6 cal deg$^{-1}$ mol$^{-1}$ for $rA_{10}$ in the 100% aqueous buffer system. These results are in reasonably good agreement with the 18.3 cal deg$^{-1}$ mol$^{-1}$ and the 23.0 cal deg$^{-1}$ mol$^{-1}$ reported by Applequist and Damle [9] for $rA_8$ and $rA_{10}$, respectively. They also agree with previously published entropy values for the disruption of A-U base pairs [13–15].

### *4.3. Solvent-induced destabilization*

Although quantitative comparisons with existing data are not possible due to the lack of previous studies of ribo-oligoadenylic acids in mixed solvent systems, the results reported here can be qualitatively discussed in terms of current views concerning the detailed nature of the forces that stabilize nucleic acid structure.

Many investigators have suggested that "hydrophobic forces" play a major role in the stabilization of double helical structures in aqueous solutions [1–5]. It is generally argued that the helical structure possesses a relatively nonpolar core environment in which the hydrophobic bases can reside. Disruption of the helical structure would force these bases to become exposed to the aqueous solvent. This would result in an ordering of the water molecules around the nonpolar bases with an attendant loss of entropy [16].

The thermodynamic data reported here do not allow us to conclude whether the solvent-induced destabilization of the $rA_8$ and $rA_{10}$ double helices is an entropic phenomenon. However, our results clearly demonstrate a decrease in the stability of the double helical structure with decreasing "aqueous character" of the solvents. In other words, the more aqueous the solvent, the more stable the double helix. In this limited sense, one can state that the helical structure is stabilized by "hydrophobic forces" without implying a particular thermodynamic interpretation. Calorimetric investigations of these transitions in the mixed solvent systems eventually will allow us to conclude whether the solvent-induced destabilization is an entropic or an enthalpic phenomenon.

It is of interest to note that the solvent-induced destabilization does not level off at mole percents as high as 15. This observation is consistent with a picture in which we are simply changing the nature of the solvent medium rather than engaging in any specific set of nucleic acid-solvent interactions which undoubtedly would reach saturation. The observed constancy of $\Delta T_m(10\%)/\Delta T_m(5\%)$ as well as the additivity exhibited by the ternary solution also are consistent with a general, non-specific solvent effect.

However, before the trends reported here can be interpreted exclusively in terms of a general, non-specific solvent effect, one must consider possible contributions from hydrogen bonding and/or electrostatic interactions.

Electrostatic effects were investigated by studying the salt-dependence of the melting temperature, $T_m$, in the different alcohol/water mixed solvents [17]. Since no significant difference was observed for the salt-dependence in pure buffer and the various mixed solvents, one can conclude that electrostatic effects do not contribute to the trends reported here; that is to say, the observed trends do not result from different electrostatic effects in the different mixed solvents.

To assess the possible contribution of H-bonding to the observed solvent-induced destabilization trends, one should compare the formamide and the DMF data. Formamide possesses two more potential hydrogen bond donor groups than DMF. Thus, if stabilization was due to hydrogen bonding between the added organic solvent and the nucleic acid bases, formamide would be a more effective denaturant than DMF. However, since the opposite effect was observed, this suggests that hydrogen bonding is not a dominant contributor to the stabilization of the helical structures.

Finally, it should be emphasized that the trends reported here are not simply due to changes in the degree of ionization of adenine in the different solvents. Tomlinson [8] investigated the possibility of such solvent-induced pK shifts for adenine and found only a small dependence of pK on solvent composition. In fact, the direction of the shifts (if they are to be considered significant) would cause an effect opposite to that of the trends that we have observed. Thus, our results cannot be ascribed to a simple solvent-induced pK shift of adenine.

With the elimination of these alternative explanations, the observed solvent-induced trends reported here can be interpreted in terms of a general, non-specific solvent effect in which increasing "aqueous character" serves to stabilize the helical structure.

In this connection, it is of interest to note that Herskovitz et al. [2] and Levine et al. [4] found similar trends for the denaturation of DNA in a series of organic/aqueous mixed solvent systems. This qualitative agreement between data obtained on two extremely different helical systems argues in favor of the general, non-specific solvent effect proposed here.

## 5. Conclusion

The results reported here indicate that relatively modest changes in the "aqueous character" of the solvent can induce dramatic changes in the stability of a helical structure by means of a general, nonspecific solvent effect. In fact, at physiological temperature (37°C) in 100% buffer solution the $rA_{10}$ double helix is stable, while in an aqueous solution to which only 5 mole percent DMF has been added, the $rA_{10}$

double helix is unstable. Thus, in proposing a mechanism for the biochemical action of a nucleic acid, one's attention should not exclusively be limited to "the most stable structure" as deduced from physical studies on the biopolymer in 100% aqueous buffer solutions.

The investigations reported here are being extended to more complex oligomeric and polymeric systems. It is the goal of such studies to provide further insights into the relative stability of nucleic acid structure under less traditional solution conditions.

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